Activation of complement at plasma-air or serum-air interface of rabbits

C. A. WARD, A. KOHEIL, D. McCULLOUGH,
W. R. JOHNSON, AND W. D. FRASER
Department of Mechanical Engineering, University of Toronto and Research Institute, Hospital for Sick Children, Toronto, Ontario M5S 1A4, Canada

WARD, C. A., A. KOHEIL, D. McCULLOUGH, W. R. JOHNSON, AND W. D. FRASER. *Activation of complement at plasma-air or serum-air interface of rabbits.* J. Appl. Physiol. 60(5): 1651–1658, 1986.—The possibility of the air-plasma interface giving rise to complement activation is investigated. After incubation of the plasma of a group of rabbits with zymosan and measurement of the degree of autologous polymorphonuclear leukocyte aggregation that follows the injection of a sample of the incubated plasma into a leukocyte suspension, it is found that the rabbits can be divided into two groups, sensitive and insensitive, depending on the degree of leukocytes aggregation. For the sensitive group it is found that both the plasma-air interface and the serum-air interface give rise to significant leukocyte aggregation. If the animal is decomplemented before the plasma is incubated in the presence of the air interface, there is no longer any significant leukocyte aggregation. It would appear that the complement system is activated by the presence of the air interface in plasma, but that fibrinogen does not play a pivotal role in the process.

bubble-blood interaction; complement activation; leukocyte aggregation

EVEN THOUGH THE BLOOD-GAS interface has been implicated in many of the events leading to damage of blood components, the mechanism by which this damage is initiated has not been established. It has been found that when a synthetic material is exposed to blood, tiny bubble nuclei can be trapped in the surface roughness of the material. These bubble nuclei can give rise to increased fibrinogen adhesion (28), platelet adhesion (9, 16, 24, 26, 27), or activation of the clotting mechanism (13, 14) and can lead to thrombus formation (17). Also, in experimental decompression sickness, evidence has been found that bubbles interact with blood components, particularly platelets and leukocytes, to produce microthrombi (2, 18–20).

The mechanism by which bubbles induce these changes has not been established. However, it is known that the presence of the fluid phase metabolites of complement activation, C3a and C5a, can give rise to both platelet and leukocyte aggregation (4, 21, 22). Thus the possibility arises that the complement system is activated by the blood-gas interface and that the complement activation metabolites which result will produce many of the changes that have been observed at the cellular level by the presence of bubbles (or bubble nuclei) in the circulatory system.

As a first step toward investigating the mechanism by which these changes could be induced, we have investigated the possibility that the bubbles activate the complement system. A necessary consequence of this activation would be that incubation of blood plasma with air bubbles would at least produce the metabolites C3a and C5a. Therefore, if a sample of the plasma incubated with bubbles were injected into a polymorphonuclear (PMN) leukocyte suspension, cell aggregation should follow (4). For human plasma, Craddock et al. (4) have introduced a method by which leukocyte aggregation induced in this fashion is used to assay for the complement metabolite C5a.

Below, we report the result of using a similar technique to assay rabbit plasma after it has been incubated either with air bubbles or with zymosan, a substance known to activate the complement system. For a group of rabbits that can be identified by their sensitivity to zymosan, it is found that incubation of their plasma with air bubbles produces a substance that aggregates its PMN leukocytes.

MATERIALS AND METHODS

Three experimental series are reported: one with rabbit plasma; one with the plasma of decomplemented rabbits; and one with the serum of normal rabbits. In preparation for an experiment a New Zealand White rabbit (~2–2.5 kg) was anesthetized by an induction dose of 3–4% halothane and then maintained on halothane at a level of 1–1.5%. Halothane was volatized with nitrous oxide (4 l/min) using the face mask method. To initiate an experiment, blood was collected from an anesthetized rabbit by the method described below.

Preparation of Plasma from Normal Rabbits

Blood was withdrawn from the femoral artery of an anesthetized rabbit into a cooled 50-ml disposable syringe containing heparin (7 IU/ml blood). Approximately 40 ml of this blood sample was centrifuged at 500 g for 10 min. Twelve milliliters of the supernatant was separated and centrifuged at 2,000 *g* for 15 min, and the latter procedure was repeated a second time. The resultant

0161-7567/86 $1.50 Copyright © 1986 the American Physiological Society

86-05330/

clear plasma was kept on ice until incubated in the presence of several air bubbles or with zymosan.

Isotonic saline was added to the blood cells collected from the first centrifugation and the remaining blood to bring the mixture to its original volume. This pooled blood was used to prepare the leukocyte suspension.

Decomplementation of Rabbits and Collection of Their Plasma

In one series of experiments rabbits were administered the cobra venom factor *Naja n. naja* (CVF^n, Cordis Laboratories, Miami, FL). This factor is known to be a potent convertase of C3 and C5 (33). On the first day of an experiment, a blood sample of 10 ml was taken from the major ear artery into a cooled syringe containing heparin (7 IU/ml blood). The plasma from this blood sample was separated by the centrifugation at 2,000 *g* and stored at −20°C until the end of an experiment. Immediately after the first blood sample was taken, the rabbit was given the first injection of CVF^n (50 IU/kg).

Approximately 24 and 48 h later this procedure was repeated, and 72 h after the first CFV^n injection a fourth blood sample of ~80 ml was taken but no CVF^n injection was given. The plasma was collected from this latter blood sample by the methods described above, and a PMN leukocyte suspension from this decomplemented animal was prepared by the methods described below.

Preparation of Rabbit Serum

Approximately 20 ml of blood were collected in a polypropylene tube from the major ear artery of a rabbit without any anticoagulant present. It was allowed to stand for at least 30 min at room temperature before being centrifuged at 2,000 *g* for 15 min. The serum was separated, and the centrifugation was repeated twice to ensure clearance of any cells or clot. The serum was then kept on ice until incubation with the different stimulants.

Preparation of the Polymorphonuclear Leukocytes

The pooled blood remaining after the preparation of the plasma from both normal and decomplemented animals was used to prepare a PMN leukocyte suspension. It was further diluted with a volume of isotonic saline equal to one-third the volume of the pooled blood, and the final heparin concentration was increased to 10 IU heparin/ml of blood. A volume of 6% dextran (BDH Chemical, Poole, UK) in saline equal to one-half the volume of the pooled blood was added to the pooled blood. The mixture was allowed to sediment at room temperature for 30 min. The leukocyte-rich portion of the plasma was separated and collected in 15 ml-polypropylene tubes for one further centrifugation at 400 *g* for 5 min at 4°C. The cell pellet was resuspended into 0.5 ml Hanks' balanced salt solution (HBSS/A, free of Ca^{2+}, Mg^{2+} and phenol red, Gibco Laboratories, Grand Island, NY) that contained 0.5% of rabbit serum albumin (Pel-Freez Biological, Rogers, AR).

The contaminating erythrocytes were lysed by the addition of 9 ml of 4°C distilled water to the cell suspension, and it was allowed to act for 25 s. Afterward, the isotonic balance was restored by the addition of 3 ml of 3.6% NaCl. The resulting cell suspension was centrifuged at 400 *g* for 5 min. The cell pellet was then resuspended in 2.5 ml HBSS/A, which was carefully placed at the top of an equal volume of Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden) solution and was centrifuged at 400 *g* for 35 min at 4°C. The PMN leukocytes that sedimented at the bottom of the tube were then suspended in 1 ml of HBSS/A, pooled, and washed twice by centrifugation before being resuspended at a concentration of 20×10^6 cell/ml in HBSS/A. The PMN leukocyte suspension was incubated with 5 μg/ml of cytochalasin B (Sigma Chemical, St. Louis, MO) at 37°C for 10 min (7). At this point the PMN leukocyte suspension was ready for use in the aggregation test.

Incubation of Plasma Samples

For each experiment, three samples of plasma were incubated.

The Plasma control. A polypropylene incubation tube with a capacity of 1.65 ml was completely filled with plasma and very carefully capped. It is essential that the tube be sealed without introducing any air bubbles. The tube was then placed in a device that held it immersed in a 37°C water bath, and it was rotated at a rate of 22 rpm, as illustrated in Fig. 1.

Plasma exposed to air interface. In another 1.65-ml polypropylene tube, 1.5 ml of plasma were added, and then the tube was capped, sealed, and agitated to fill the remaining volume with a number of bubbles. Afterward it was also placed in the rotation device, along with the plasma control. The rotation was such that the air bub-

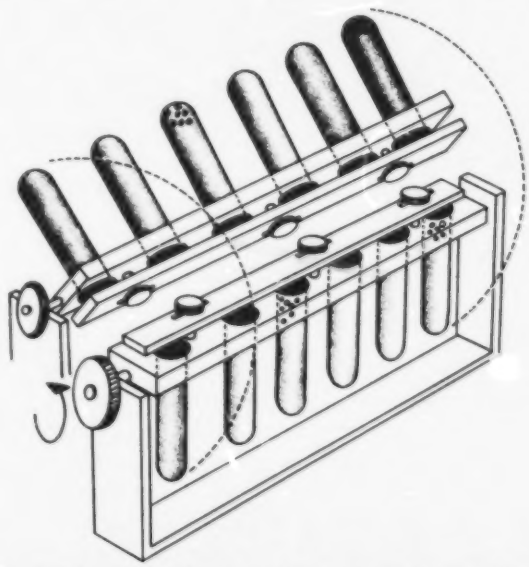

FIG. 1. Schematic of device used to incubate plasma and serum samples. Note that rotation is such that air bubbles in a plasma or serum sample traverse length of incubation tube in each half rotation. This device was placed in a water bath maintained at 37°C and rotated during incubation.

bles traversed the length of the tube twice during each rotation (see Fig. 1).

On some occasions, the air volume in the incubation tube was left as a single bubble, but it was found that it would break into a number of smaller bubbles during the rotation.

Zymosan-activated plasma. In each experiment, a third sample of plasma (1.5 ml) was added to a polypropylene incubation tube along with 7.5 mg zymosan (Sigma Chemical). This plasma sample was also placed in the rotating device and incubated along with the other two samples for 30 min. Afterward, the plasma sample that had been incubated with zymosan was centrifuged at 2,500 *g* for 10 min, and the clear supernatant was separated and placed in a clean tube for the aggregation test. It and the other plasma samples were placed in an ice bath and held there until they were assayed with the aggregometry technique described below.

Incubation of Serum Samples

In a number of our experiments, three samples of serum were incubated in addition to those samples of plasma described above. The first sample of serum was incubated in the absence of any added stimulant to complement activation and served as the control; the second one was incubated in the presence of air bubbles and the third in the presence of zymosan. The incubation procedure and container material for the serum were the same as those for the plasma control.

Assay for Complement Activation

Two methods were used to assay for complement activation. The PMN leukocyte aggregation procedure (3–5, 7, 25) was used to assay the plasma and serum from both the normal animals and the animals decomplemented with CVF^n. For the animals receiving the CVF^n on successive days, the blood samples were also assayed for C3 using the rocket immunoelectrophoresis (31).

PMN leukocyte aggregation assay. The PMN leukocyte aggregation procedure that we adopted is based on earlier work (4, 5, 7, 25). In summary, 0.45 ml of PMN leukocyte preparation was pipetted into a siliconized glass cuvette and was placed in an aggregometer where the contents were stirred magnetically at 800 rpm and maintained at 37°C. After equilibration of the PMN leukocyte suspension, a sample of incubated plasma or serum was added to the suspension. A change in the light transmittance of the suspension, due to cell aggregation, was recorded on a strip-chart recorder for 3 min after the addition of an incubated plasma or serum sample. The measurements were made at 30, 45, and 60 s after the addition of the incubated plasma or the serum sample. The change in the light transmission was taken as a measure of the activated complement in a sample. The reason for this is discussed below.

Rocket immunoelectrophoresis. For the group of rabbits subjected to the decomplementation procedure, samples of their plasma were assayed for the presence of C3 using rocket immunoelectrophoresis (RIE) before CVF^n was administered and during the decomplementation process. The RIE technique adopted was that described by Laurell (10), and involves the following.

Agarose M powder was suspended in tris(hydroxymethyl)aminomethane (Tris)barbiturate buffer (pH 8.6, LKB-Produkter, Bromma, Sweden) at a concentration of 1% and was heated with constant stirring until melted. The gel was cooled to ~55°C, and rabbit C3 antibody was added (0.8 μl/ml gel, from U.S. Biochemical, Cleveland, OH). After mixing the gel and C3 antibodies, they were cast onto a gel-bond film (FMC, Marine Colloids Div., Rockland, ME). The gel film was left to harden at room temperature and then stored at 4°C until used. After wells were made in the gel film for sample application, the gel film was placed on the cooling plate of the LKB 2117 Multiphor electrophoresis apparatus (LKB-Produkter).

Ten-microliter plasma samples and titer dilutions were made with the Tris-barbiturate buffer and applied in the wells while the gel film was on the cooling plate. The gel film was subjected to electrophoresis for 16–18 h at 6–8°C with an electric field strength of 5 V/cm.

After the electrophoresis was completed, the gel film was removed and washed three times with distilled water using filter paper as a fluid absorbent for a period of 3 min. A second wash with saline was repeated twice, and the absorbent paper was left on the gel film for 15 min to remove any nonbound proteins. The gel film was finally dried in a hot air in an oven. It was then stained in 0.5% Coomassie blue (G. T. Gurr, London, UK) for 5 min and destained with ethanol-acetic acid-water (9:1:9) solution and dried. Finally, the peak height of the rocket-shape antibody-antigen precipitate was measured and recorded.

EXPERIMENTAL PROCEDURES AND RESULTS

Complement Assay Procedure Using PMN Leukocyte Suspension

The PMN leukocyte aggregation procedure described above was introduced by Craddock et al. (4), and for human plasma it has been found to be a sensitive method to assay for the complement metabolite C5a (4, 5, 7, 25), although it does not provide a quantitative technique (7). (The change in light transmission increases with the log of the concentration of C5a in the plasma sample).

For rabbit plasma, it is possible that a portion of the change in light transmission is produced by the presence of the complement metabolite C3a, since it has chemotactic properties as well (23, 29, 30).

Using the plasma collected from normal rabbits and incubated with the plasma-air interface present, we formed a titer of three-fourth dilutions with the dilutions made from HBSS/A buffer. For the five dilutions from full strength to 1:81/256, the change in light transmission was linear with the concentration of incubated plasma. We have previously shown that the change in light transmission of a series of double dilutions of rabbit plasma incubated with zymosan also shows a linear dependence on incubated plasma concentration (25).

We shall assume that the change in light transmission

following the injection of an incubated plasma sample taken from a rabbit into a suspension of its own PMN leukocytes is related to the amount of activated complement (C3a and/or C5a). This assumption is discussed further below.

Effect of Decomplementation Procedure on Presence of C3 in Rabbit Plasma

In Fig. 2, the results of a RIE experiment are shown. The first six peaks were obtained from a titer of plasma incubated without any added stimulant, i.e., from the plasma control sample. The dilutions were made with the Tris-barbiturate buffer. The first peak corresponds to a dilution of 1:4 and the last 1:128. All other peaks (i.e., *7–18*) were made from plasma diluted in the ratio of 1:4. *Peaks 7* and *8* were made from the plasma incubated with air bubbles, *peaks 9* and *10* from plasma incubated with zymosan, and *peaks 11* and *12* from the plasma control. The other peaks were obtained from plasma taken 1 day after the first injection (*peaks 13* and *14*) and show the effect of the first injection; *peaks 15* and *16* were taken 2 days afterward and show the effect of the first two injections; and the last two peaks were made from plasma taken 3 days after and show the effects of three injections.

The peak height would be a measure of the amount of C3 present in the plasma. Thus the indication from this would be that the concentration of C3 in the plasma 24 h after the first injection of CVF^n is reduced to ~3% of its normal value in plasma (i.e., compare *peak 4* with *peak 13* and *14*). Since C3 is essential for complement activation by either the classical or the alternative pathways, one could expect little complement activation in the plasma samples taken from the rabbit either 48 or 72 h after the first injection and incubated with different stimulants to complement activation.

Examination by PMN Leukocyte Aggregation of Decomplemented Plasma Incubated with Different Stimulants

The plasma collected from decomplemented rabbits and incubated with different stimulants was examined with the PMN leukocyte aggregation test. The results are summarized in Fig. 3.

The plasma collected before the decomplementation process was started and at three times during it was stored until 72 h after the first injection of CVF^n. A portion of blood collected at 72 h was divided into two portions. One portion was used to prepare plasma samples to be incubated with different stimulants, and the other portion to prepare a PMN leukocyte suspension. The plasma samples collected before the first injection and during the decomplementation were divided into three portions. One portion of the plasma was incubated with zymosan, one portion with the plasma-air interface present (i.e., with air bubbles), and one portion without any stimulant added. The latter plasma samples served as controls. Samples of the plasma that had been incubated with each of the stimulants were then assayed for complement activation using the PMN leukocyte suspension by the method described above.

The measured change in light transmission observed from a sample of plasma incubated with the different stimulants was divided by that measured from the control sample. This ratio is plotted as the ordinate in Fig. 3. The solid line was obtained from the samples incubated with zymosan and the dashed line from the samples incubated in the presence of the plasma-air interface. The abscissa in Fig. 3 represents the times after the first injection of CVF^n. When the values of these ratios are both unity, there would be no measured complement activation by the PMN leukocyte aggregation technique. Note that there is no measured complement activation in either the samples incubated with zymosan or in those

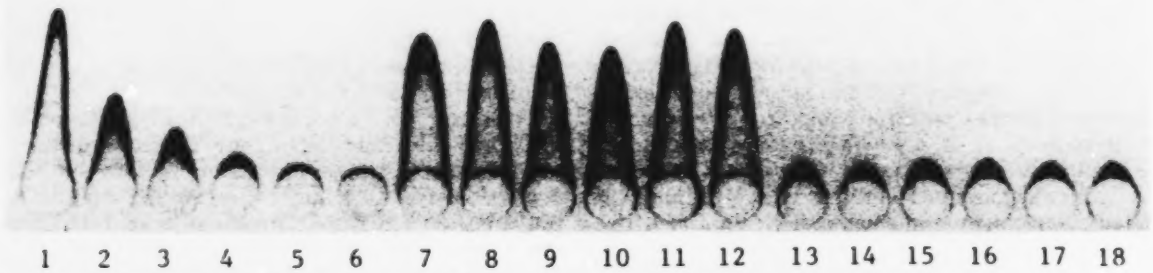

FIG. 2. Rocket immunoelectrophoresis assay for C3 in plasma of a normal rabbit and of its plasma when rabbit was being decomplemented. *Peaks 1–6* are titers of normal plasma; *7–10* are of normal plasma incubated with different stimulants to complement activation; *11* and *12* are of normal rabbit; *13* and *14* were taken after 1st stage of decomplementation, *15* and *16* after 2nd stage, and *17* and *18* were after 3rd stage of decomplementation with cobra venom factor *Naja n. naja*.

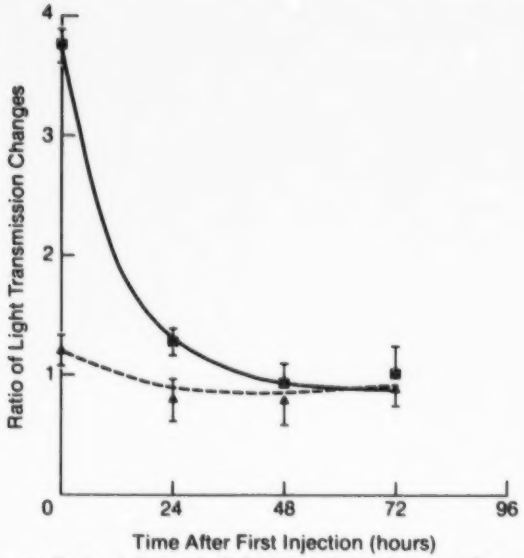

FIG. 3. Ratio of change in light transmission from a polymorphonuclear leukocyte suspension formed from cells of a decomplemented animal. Ratio was obtained from change in light transmission following an injection of plasma incubated with a stimulant divided by change in light transmission from a control sample. For *solid line*, stimulant was zymosan; for *dashed line*, it was plasma-air interface.

incubated in the presence of the air interface 48 or 72 h after the first injection of CVF^n. The results presented in Fig. 3 are based on averages from three experiments.

Complement Activation at Plasma-Air Interface of Normal Rabbits

In each experiment of this series, plasma obtained from the normal rabbits by the methods described above was incubated with two stimulants. One stimulant was zymosan and the other was the plasma-air interface. A third plasma was incubated without any agent added and served as the control. The PMN leukocyte aggregation test was used to assay the incubated plasma samples for complement activation. The change in light transmission was taken as a measure of the activated complement.

A wide variation was found between the sensitivity of individual rabbits to complement activation by zymosan. The animals were therefore classified into two groups: those that were "sensitive" to zymosan and those that were not. A rabbit was classified as being sensitive to zymosan if the measured complement activation in the plasma sample incubated with this stimulant divided by that measured in the plasma control sample was three or more. This ratio is denoted as R and the reason for choosing the value of three for classifying a rabbit as sensitive is discussed further below.

The results obtained from the differently classified animals are shown in Fig. 4. The ordinate in Fig. 4 was obtained from the complement activation measured in the control sample of the plasma (labeled C), and the sample incubated with the plasma-air interface present (labeled PAI) and expressed as a percentage of that measured for the plasma sample incubated with zymosan.

The measured complement activation in the plasma

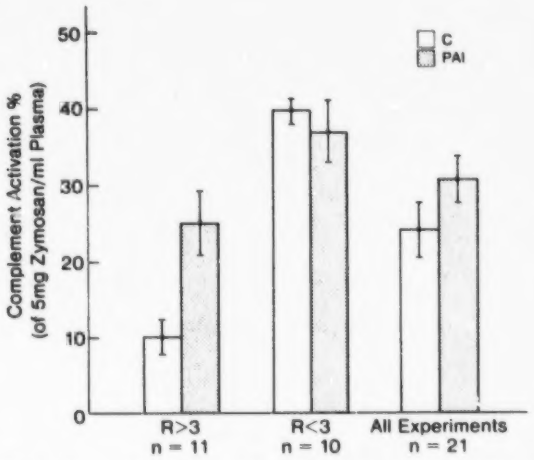

FIG. 4. Measured complement activation by plasma-air interface (PAI). Rabbits have been divided into 2 groups: those for which ratio (R) of measured complement activation in plasma samples incubated with zymosan was at least 3 times that measured in control samples (C); i.e., $R \geq 3$, and those for which this ratio is less; i.e., $R < 3$. *Bars*, SE.

samples incubated in the presence of the plasma-air interface and the plasma control for the individuals satisfying the criterion of being sensitive to zymosan are plotted as the first pair of bars in Fig. 4. The results obtained for those animals not satisfying this criterion are shown by the second pair of bars. The third bar pair is for all of the experiments in which the complement activation at the plasma-air interface was measured.

When examined for statistical significance with Student's paired t test, it is found that the group of animals that were sensitive to zymosan were also sensitive to complement activation at the plasma-air interface. For this group, the complement activation in the plasma samples incubated with the air bubbles was statistically greater than that in the control samples at the 99% level (i.e., $P < 0.01$ for the first bar pair in Fig. 4). Note that ~50% of the animals tested were classified as sensitive to zymosan.

For the group of animals that were not sensitive to zymosan (i.e., for those with $R < 3$), there was no statistical significance to the difference between the mean of the measured complement activation in the plasma sample incubated with the air bubbles and that of the controls (see the second bar pair in Fig. 4).

When the means obtained for all animals were examined, it was also found that the complement activation in the plasma samples incubated with the air bubbles were statistically greater than those of the controls at the 95% level. These latter results are shown as the third bar pair in Fig. 4.

To examine the criterion chosen for classifying animals as sensitive or insensitive to complement activation by zymosan, the effect of other choices for the value of R on the conclusions has been examined. The results obtained by choosing R to be 3, 6, and 10 are shown in Fig. 5. In each case, the plasma samples incubated with air bubbles were found to have a statistically greater

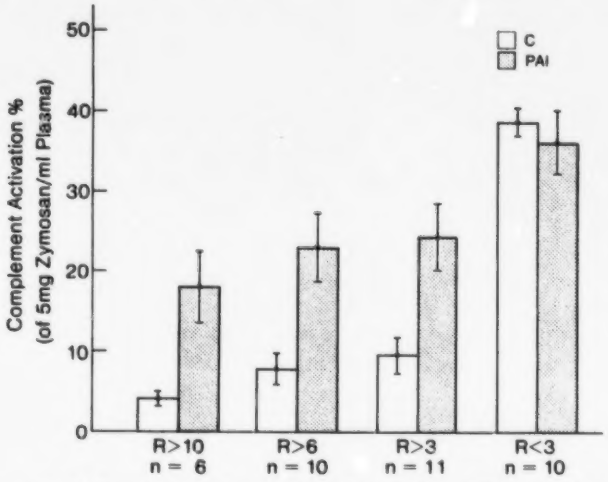

FIG. 5. Examination of conclusions obtained from different choices for ratio (R) of complement activation in plasma samples incubated with zymosan compared with that in control samples. Results obtained from plasma controls are labeled C and those obtained from plasma incubated with air bubbles as PAI. *Bars*, SE.

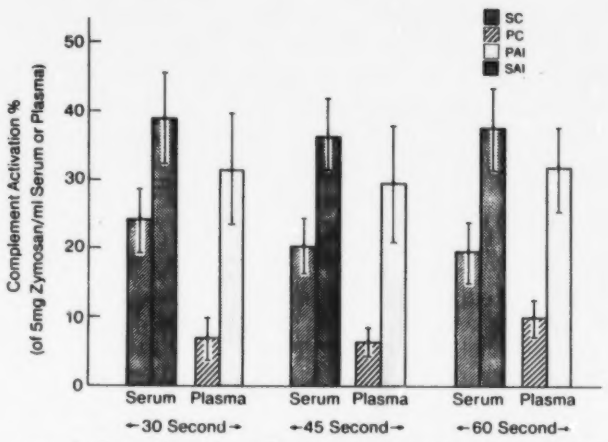

FIG. 6. Comparison of complement activation at serum-air interface (SAI) with that at plasma-air interface (PAI). Time was measured from instant that incubated sample was injected into polymorphonuclear leukocyte suspension. Results obtained for plasma control and serum control are denoted by PC and SC, respectively. *Bars*, SE.

amount of activated complement than did the controls at the 95% level on Student's paired *t* test. For those animals for which R was <3, there was no statistically significant difference in complement activation between those plasma samples incubated in the presence of the air bubbles and the controls.

Complement Activation at the Serum-Air Interface

For a series of five experiments with normal rabbits, both plasma and serum from the same animal were prepared and samples of each of them were incubated with the stimulants zymosan and the air interface. Also, one tube was filled with plasma and one with serum in each experiment, and they were incubated without any additional stimulant. These latter samples served as the controls. In this group of experiments, four of the five individual rabbits exhibited complement systems that were sensitive (R > 3). In Fig. 6, we show the measured complement activation in both plasma and serum for these five experiments. The complement activation measured in the respective control samples and in those samples incubated with the air interface were expressed as percentages of the complement activation measured in the respective samples incubated with zymosan. These values are given as the ordinate in Fig. 6. The measurements are reported at three different times after the injection of the activated plasma or serum into the PMN leukocyte suspension.

The results shown in Fig. 6 were also examined for statistical significance using Student's paired *t* test. For the measurements made at 30 s, the complement activation in the samples containing the serum-air interface was statistically greater than the serum control at the 80% level and at the 90% or greater level at all other times. The samples containing the plasma-air interface were statistically greater than the plasma controls at the 95% level at all times measured.

DISCUSSION

The PMN leukocyte aggregation technique has been found to be a sensitive measure of the presence of C5a in human plasma samples (3–5, 7). For rabbit plasma this test may indicate the presence of C3a, as well as C5a, since the former complement metabolite has chemotactic properties (23, 29, 30). However, we note that none of the other plasma enzyme systems of rabbit plasma, such as activated Hageman factor, kallikrein, high-molecular-weight kininogen is capable of producing chemotactic activity without the presence of C5, which they can convert to C5a (32), nor does intact C5 produce chemotactic activity (32). Thus it would appear that the PMN leukocyte aggregation following the injection of an incubated plasma sample would result from the presence of either or both of these two complement metabolites. We do not try to distinguish between the presence of C3a or C5a in the work we report here.

The experiments with decomplemented rabbits would support the assumption that the PMN leukocyte aggregation test reacts to activated complement only. The results in Fig. 3 show that there is no longer any measured difference between the controls and either the zymosan-incubated plasma or the plasma incubated with air bubbles after two injections of CVF^n, as measured with the PMN leukocyte aggregation test. The CVF^n is a strong convertase of C3 and would thus effectively remove the ability of either of these stimulants to produce activated complement by either the classical or alternative pathways. The results shown in Fig. 2 indicate that, after the two injections CVF^n, there is little C3 present in the plasma sample. Therefore, if our assumption of PMN leukocyte aggregation being related to the presence of C3a and C5a in the plasma sample were valid, we would expect to see no difference in the reading for the controls and these two potential stimulants of complement activation. This is the observed result at 48 and 72 h (see Fig. 3). This result would indicate that an intact complement system is necessary for the plasma-

air interface to produce complement activation as measured by this test, and it shows that the PMN leukocyte aggregation test gives the null result when there is no complement activation. The linear titer test shows that the change in light transmission is related to the (log of) concentration of the activated complement components in the plasma of normal rabbits incubated with air bubbles (25).

As illustrated in Figs. 4–6, the PMN leukocyte aggregation test indicates that for all normal rabbits examined, there is some sensitivity to complement activation in plasma or serum incubated with zymosan. However, the degree of this sensitivity varied greatly among the rabbits.

A striking feature of the results illustrated in Fig. 4 is that those rabbits that were sufficiently sensitive to zymosan (i.e., those for which $R > 3$) were also sensitive to complement activation by the plasma-air interface. Thus those rabbits that are sensitive to complement activation at the plasma-air interface can be identified by their sensitivity to zymosan. Note that the complement activation at the plasma-air interface for the sensitive group of rabbits is >2.5 times that of the controls (see the first bar pair of Fig. 4).

We note that the experiments reported in Fig. 3 for the animals undergoing decomplementation did not display the same degree of sensitivity to the plasma-air interface (see the results at *time 0* in Fig. 3) as the normal animals, but this may have been because the cells from the decomplemented animal were less sensitive or because of the difference in experimental technique (see above). The plasma sample taken before the decomplementation process was begun (i.e., *time 0*) was not tested until more than 72 h later.

The results shown in Fig. 5 indicate that, provided one chooses to classify an animal as being sensitive to zymosan when R for the animal is 3 or greater, one reaches the same conclusion: if the animal is sensitive to zymosan, it is also sensitive to complement activation at the plasma-air interface. Choosing R to be 3 as the criterion for sensitivity to zymosan appears to be the lower limit for which this conclusion remains valid (see the last pair of bars in Fig. 5).

The mechanism by which the plasma-gas interface could activate the complement system is not clear. One possibility is that the plasma-air interface acts as a surface on which adsorption of another agent takes place and the adsorbed agent acts as the surface that initiates the activation of the complement system. Studies of the blood-bubble interface have indicated that a film forms at the interface that probably consists of glycoproteins (12). Fibrinogen is one such protein, and it has been found to be strongly adsorbed at the blood-gas interface (9). Furthermore, it has been observed that the formed elements of blood are damaged when blood is oxygenated by bubbling (i.e., with a bubble oxygenator), and it has been hypothesized that this damage results from processes that are initiated by the denaturation of fibrinogen at the blood-bubble interface (1, 11). It would therefore be reasonable to expect that fibrinogen, adsorbed and denatured at the plasma-air interface, could be responsible for the observed complement activation at the plasma-air interface that we have observed.

This proposal can be examined to some degree by the results reported in Fig. 6 where the complement activation in serum as well as in plasma from the same animal is reported. The measured complement activation in the serum samples with serum-air interface present is labeled SAI in Fig. 6. Note that at each measurement time, the serum-air interface was found to give rise to complement activation, as can be seen from the fact that the complement activation in the serum sample incubated in the presence of the serum-air interface was larger than the complement activation in the control sample of serum. Thus, even though a great deal of the fibrinogen was removed in the preparation of the serum, the serum-air interface nonetheless activates the complement system. This would indicate that fibrinogen does not play an essential role in the activation of the complement system by the plasma-air interface.

In a population of normal rabbits, a significant fraction produces a substance that leads to PMN leukocyte aggregation when either their plasma or their serum is incubated with air bubbles. The sensitive group can be identified independently by their response to incubation of their plasma with zymosan. The PMN leukocyte aggregation that results from incubating their plasma with air bubbles can be prevented if the animal is decomplemented before the incubation is performed. Since none of the other plasma enzyme systems are capable of producing chemotactic effects without producing either C3a or C5a, the indication is that the complement system is activated at either the plasma-air or the serum-air interface. The fact that complement activation takes place at both these interfaces would indicate that fibrinogen (perhaps damaged at the plasma-air interface) does not play an essential role in the complement activation process.

This work was supported by the Ontario Heart Foundation, Canada, and by the Defense and Civil Institute of Environmental Medicine under Contract 01SE.97711-5-8632, Paper 86-P-03.

Received 24 September 1984; accepted in final form 19 November 1985.

REFERENCES

1. ASHMORE, P. G., V. SRITCK, AND P. AMBROSE. The incidence and effects of particulate aggregation and microembolism in pump oxygenator system. *J. Thorac. Cardiovasc. Surg.* 55: 691–697, 1968.
2. CLARK, M. L., R. B. PHILP, AND C. W. GOWDEY. Changes in platelets and lipids in experimental aeroembolism and bends. *Aerosp. Med.* 40: 1094–1098, 1969.
3. CRADDOCK, P. R., J. FEHR, A. P. DALMASSO, K. L. BRIGHAM, AND H. S. JACOB. Hemodialysis leukopenia. *J. Clin. Invest.* 59: 879–888, 1977.
4. CRADDOCK, P. R., D. HAMMERSCHMIDT, J. G. WHITE, A. P. DALMASSO, AND H. S. JACOB. Complement (C5a)-induced granulocyte aggregation in vitro. *J. Clin. Invest.* 60: 260–264, 1977.
5. CRADDOCK, R. B., J. G. WHITE, AND H. S. JACOB. Potentiation of complement (C5a)-induced granulocyte aggregation by cytochalasin B. *J. Lab. Clin. Med.* 91: 490–499, 1978.
6. GOLDING, F. C., P. D. GRIFFITHS, W. D. M. PATON, D. N. WALDER, AND H. V. HEMPLEMAN. Decompression sickness during the construction of the Danford Tunnel. *Br. J. Ind. Med.* 17: 167–180, 1960.
7. HAMMERSCHMIDT, D. E., T. K. BOWERS, C. J. LAMMI-KEEFE, H. S. JACOB, AND P. R. CRADDOCK. Granulocyte aggregometry: a sensitive technique for the detection of C5a and complement acti-

vation. *Blood* 55: 898–902, 1980.

8. HEMPLEMAN, H. V. History of evolution of decompression procedures. In: *The Physiology and Medicine of Diving* (3rd ed.), edited by P. B. Bennett and D. H. Elliott. San Pedro, CA: Best, 1978, p. 319.
9. KOLOBOW, T., AND T. TOMLINSON. Vacuum priming of the membrane lung: a new and rapid technique. *Cardiovasc. Pulmon. Tech.* 5: 18–19, 1977.
10. LAURELL, C. B. Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. *Anal. Biochem.* 15: 45–52, 1966.
11. LEE, W. H., JR., AND P. HAIRSTON. Structural effects on blood proteins at the gas-blood interface. *Federation Proc.* 30: 1615–1622, 1971.
12. LEHTO, V. P., I. KANTOLA, T. TERVO, AND L. A. LAITINEN. Ruthenium red staining of blood-bubble interface in acute decompression sickness in rats. *Undersea Biomed. Res.* 8: 101–111, 1981.
13. MADRAS, P. N., W. R. JOHNSON, AND C. A. WARD. Enhancement of thrombus formation by vascular prostheses. *Can. J. Surg.* 23: 544–546, 1980.
14. MADRAS, P. N., C. A. WARD, AND W. R. JOHNSON. Enhanced thromboresistance of surfaces by denucleation. *Trans. Am. Soc. Artif. Intern. Organs* 26: 153–158, 1980.
15. NISHI, R. Y., K. E. KISMAN, B. C. EATOCK, I. P. BUCKINGHAM, AND G. MASUREL. Assessment of decompression profiles and divers by Doppler ultrasonic monitoring. In: *Proceedings of the Seventh Symposium on Underwater Physiology*, edited by A. J. Bachrach and M. M. Matzer. Bethesda: MD: Undersea Med. Soc., 1981, p. 717–728.
16. OSADA, H., J. DUFFIN, C. A. WARD, J. M. NELMS, AND J. D. COOPER. Elimination of gas nuclei from membrane oxygenator surface during priming improves platelet preservation. *Artif. Organs* 2: 121–125, 1977.
17. OSADA, H., C. A. WARD, J. DUFFIN, J. M. NELMS, AND J. D. COOPER. Microbubble elimination during priming improves biocompatibility of membrane oxygenators. *Am. J. Physiol.* 234 (*Heart Circ. Physiol.* 3): H646–H652, 1978.
18. PHILP, R. B. A review of blood changes associated with compression-decompression: relationship to decompression sickness. *Undersea Biomed. Res.* 1: 117–150, 1974.
19. PHILP, R. B., C. W. GOWDEY, AND M. PRASAD. Changes in blood lipid concentration and cell counts following decompression sickness in rats and the influence of dietary lipid. *Can. J. Physiol. Pharmacol.* 45: 1047–1059, 1967.
20. PHILP, R. B., M. M. INWOOD, AND B. A. WARREN. Interactions between gas bubbles and components of the blood: implications in decompression sickness. *Aerosp. Med.* 43: 946–953, 1972.
21. POLLEY, M. J., AND R. L. NACHMAN. The human complement system in thrombinmediated platelet function. *J. Exp. Med.* 147: 1713, 1978.
22. POLLEY, M. J., AND R. L. NACHMAN. Human platelet activation by C3a and C3a des arginine. *J. Exp. Med.* 158: 603–615, 1983.
23. SNYDERMAN, R., H. GEWURZ, AND S. E. MERGENHAGEN. Interactions of the complement system with endotoxic polysaccharide. Generation of a factor chemotactic for polymorphonuclear leukocytes. *J. Exp. Med.* 128: 259–275, 1968.
24. WARD, C. A., AND T. W. FOREST. On the relation between platelet adhesion and roughness of a synthetic biomaterial. *Ann. Biomed. Eng.* 4: 184–207, 1976.
25. WARD, C. A., A. KOHEIL, W. R. JOHNSON, AND P. N. MADRAS. Reduction in complement activation from biomaterials by removal of air nuclei from the surface roughness. *J. Biomed. Mater. Res.*, 18: 255–269, 1984.
26. WARD, C. A., B. RUEGSEGGER, D. STANGA, AND W. ZINGG. Reduction in platelet adhesion to biomaterials by removal of gas nuclei. *Trans. Am. Soc. Artif. Intern. Organs* 9: 77–84, 1975.
27. WARD, C. A., B. RUEGSEGGER, D. STANGA, W. ZINGG, AND M. A. HERBERT. Relation of proteins, platelets, and gas nuclei in adhesion to a synthetic material. *Am. J. Physiol.* 233 (Heart Circ. Physiol. 2): H100–H105, 1977.
28. WARD, C. A., D. STANGA, B. J. ZDASIUK, AND F. S. GATES. Effect of air nuclei on the adsorption of fibrinogen to silicon rubber. *Ann. Biomed. Eng.* 7: 451–469, 1979.
29. WARD, P. A. A plasmin-split fragment of C3 as a new chemotactic factor. *J. Exp. Med.* 126: 189–206, 1967.
30. WARD, P. A., C. G. COCHRANE, AND H. J. MULLER-EBERHARD. Further studies on the chemotactic factor of complement and its formation in vivo. *Immunology* 11: 141–153, 1966.
31. WEEKE, B. Rocket immunoelectrophoresis. *Scand. J. Immunol.* 2: 37–46, 1973.
32. WIGGINS, R. C., P. C. GICLAS, AND P. M. HENSON. Chemotactic activity generated from the fifth component of complement by plasma kallikrein of the rabbit. *J. Exp. Med.* 153: 1391–1404, 1981.
33. ZABERN, I. V., B. HINSCH, H. PRZYKLENK, G. SCHMIDT, AND W. VOGT. *Immunobiology* 157: 499–514, 1980.